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Purification and use of *E. coli* peptide deformylase for peptide deprotection in chemoenzymatic peptide synthesis

Claudia Di Toma [a], Theo Sonke [a], Peter J. Quaedflieg [a], Dick B. Janssen [b,*]

[a] DSM Innovative Synthesis, P.O. Box 18, 6160 MD Geleen, The Netherlands
[b] Department of Biochemistry, Groningen Biomolecular Science and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

Peptide deformylases (PDFs) catalyze the removal of the formyl group from the N-terminal methionine residue in nascent polypeptide chains in prokaryotes. Its deformylation activity makes PDF an attractive candidate for the biocatalytic deprotection of formylated peptides that are used in chemoenzymatic peptide synthesis. For this application it is essential to use PDF preparations that are free of contamination by peptidases that can cleave internal peptide bonds. Therefore, different purification methods were attempted and an industrially applicable purification procedure was developed based on a single anion-exchange chromatography step of an engineered PDF variant that was equipped with an anionic octaglutamate tag. The deformylation activity and stability of the engineered enzyme were similar to those of the wild-type PDF. This purification method furnished a PDF preparation with a 1500-fold decreased level of contamination by amidases and peptidases as compared to cell-free extract. It was shown that the enzyme could be used for deprotection of a formylated dipeptide that was prepared by thermolysin-mediated coupling.

Introduction

Chemical peptide synthesis, either in solution or by solid phase methods, generally proceeds by elongation at the N-terminal side of the nascent peptide chain. In academic and industrial practice *N*-protecting groups like *tert*-butoxycarbonyl (Boc)[1], benzyloxycarbonyl (Cbz) and especially fluorenylmethoxycarbonyl (Fmoc) are used in combination with advanced coupling reagents such as carbodiimides, phosphonium salts, or uronium/guanidinium salts [1]. Examples of coupling reagents are 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/HOSu), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxybenzotriazole (EDC/HOBt) or benzotriazolyloxy-tris-(pyrrolidino)-phosphonium hexafluorophosphate (ByBOP) [2]. Although these coupling methods work well on a laboratory scale and proceed without substantial racemisation of the amino acid building blocks, cheaper protecting groups and coupling reagents are desired for large scale industrial application. In this respect, a particularly interesting N-terminal amino protecting group is the formyl moiety, which can be readily introduced at low cost using formic acid and acetic acid anhydride [3]. The removal of the N-terminal formyl group after the coupling reaction can be performed chemically or enzymatically. Acidic hydrolysis is a conventional method for removing *N*-formyl groups from amino acids and from the amino terminus of peptides [4]. However, the low pH and harsh reaction conditions lead to significant peptide bond hydrolysis when this method is applied in peptide synthesis. A mild and selective enzymatic method would thus be more attractive.

A class of enzymes that can be used for the removal of *N*-formyl-protecting groups from peptides consists of the peptide deformylases. The cellular ribosome-mediated synthesis of proteins starts with a methionine residue. In prokaryotes and eukaryotic organelles (mitochondria and chloroplasts), the methionyl moiety carried by the initiator tRNA. fMet is *N*-formylated prior to its incorporation into a polypeptide [5]. Following initiation of translation, the enzyme peptide deformylase (PDF, EC 3.5.1.88) cleaves the formyl group from the nascent polypeptide chain [6]. Next, methionyl aminopeptidase may remove the N-terminal methionine from the deformylated polypeptide, leading to a mature protein [7]. Because of this deformylase activity, PDF can be an attractive biocatalyst for the deprotection of formylated peptides.

* Corresponding author. Fax: +31 50 3634165.
E-mail address: d.b.janssen@rug.nl (D.B. Janssen).

[1] *Abbreviations used:* PDF, peptide deformylase; EcPDF, *E. coli* peptide deformylase; CFE, cell-free extract; Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; EDC/HOSu, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide; EDC/HOBt, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxybenzotriazole; ByBOP, benzotriazolyloxy-tris-(pyrrolidino)-phosphonium hexafluorophosphate; IEC, ion-exchange chromatography; TCEP, tris-(2-carboxyethyl)-phosphine; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethyl sulfoxide, NMP, N-methyl-2-pyrrolidinone.

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Although much knowledge is available on PDFs as a target for antibacterial, antiparasitic and chemotherapeutic agents [8–10], data on biocatalytic applications are scarce. Concerning the use of PDF in organic synthesis, the only information available in the literature describes the application of the enzyme in the synthesis of enantiopure amines and amino acid derivatives starting from racemates, based on its ability to hydrolyze *N*-formyl α-aminonitrile and *N*-formyl derivatives of non-functionalized amines and β-amino alcohols in a stereoselective manner [11]. In this paper, the authors also describe an example of the use of PDF for the selective deprotection of *N*-formyl-dipeptides. A prerequisite of the application of PDFs in peptide synthesis is the possibility to isolate the enzyme in a form that is free of contaminating peptidases that may originate from the *E. coli* host strain used for overexpression and could cause peptide bond hydrolysis in the peptide that is under synthesis.

The aim of the work described here is to obtain a method for the rapid, upscalable, and cheap production of purified PDF that is capable of removing N-terminal formyl groups from peptides without concurrent peptide bond hydrolysis. A one-step purification method for PDF based on affinity chromatography with a Met–Lys modified Sepharose matrix was described before [11]. This strategy is in practice only suitable for use on laboratory scale since the matrix is not commercially available and its preparation involves a complicated chemo-enzymatic preparation process making use of expensive materials. In this paper, we explore the development of two simple and industrially applicable purification methods for peptide deformylase from *E. coli* (EcPDF).

Because EcPDF is a monomeric enzyme of 168 aa (19.24 kDa), which is rather small as compared to the average *E. coli* amidases and peptidases, the use of ultrafiltration methods was first examined. Membrane ultrafiltration (UF) is a pressure-modified, convective process that uses semi-permeable membranes to separate species in aqueous solutions by molecular size, shape and/or charge [12]. Alternatively, the selectivity of ion-exchange chromatography (IEC) may be improved by modification of the target protein, e.g. by changing the charge distribution on the surface [13]. The second option that is described makes use of IEC of a variant of PDF that is equipped with a negatively charged octaglutamate tag introduced in a solvent exposed loop on the surface of EcPDF. This allowed efficient purification using IEC.

Experimental

Materials

All standard chemicals were of the highest grade obtainable. H-Phe-NH_2, H-Tyr-OMe, *N*-formyl-Met–Ala-OH, H-Met–Ala-OH, *N*-formyl-Met–Lys-OH, H-Leu–Phe-OH, H-Phe–Leu-OH, H-Asp–Phe-OH, H-Phe–Asp-OH, H-Arg–Phe-OH, H-Phe–Arg-OH, H-Gly–Phe-OH, and H-Phe–Gly-OH were purchased from Bachem (Bubendorf, Switzerland); tris-(2-carboxyethyl)-phosphine (TCEP) was obtained from Fluka (Buchs, Switzerland). If desired, amine groups were formylated in a refluxing mixture containing 1.1 equiv formic acid and 1.1 equiv of acetic acid anhydride [11].

The mono Q HR 5/5 and HiLoad Q Sepharose anion exchange columns were purchased from GE Healthcare Bio-Sciences Ltd (United Kingdom). Centriprep and Centricon centrifugal filter devices were purchased from Millipore Corporation (Billerica, Massachusetts, USA).

E. coli strains CJ236 and JM109 were from Bio-Rad Laboratories GmbH (Munich, Germany). Helper phage M13K07 was from GE Healthcare Bio-Sciences Ltd (United Kingdom).

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and polynucleotide kinase were obtained from New England Biolabs (Schwalbach, Germany). Adenosine triphosphate and deoxynucleotide triphosphates were purchased from Roche (Mannheim, Germany). Catalase from bovine liver was obtained from Sigma (St. Louis, Missouri, USA). Synthesis of oligonucleotides for site-directed mutagenesis and DNA sequencing were performed by MWG Biotech AG (Ebersberg, Germany).

Construction of *E. coli* PDF wild-type expression vector

Wild-type peptide deformylase from *E. coli* ($EcPDF_{wt}$) was produced with the expression vector pBAD/*Myc*-His-DEST using Gateway cloning technology (Invitrogen). The PDF gene was first amplified by PCR using forward primer 5′-GGG*GACAAGTTTGTACAAAAAAGCAGGCT*AGG-AGGAATTAACCA**ATG**TCCGTGCTTCAAGTGT TACATATTCC-3′ as (*attB1* site in italics, Shine–Dalgarno sequence underlined, start codon in boldface), and reverse primer 5′-GGG *GACCACTTTGTACAAGAAAGCTGGGT***TTA**AGCCCGGGCTTTCAGACGATC CAGTTTTTC-3′ as (*attB2* site in italics, stop codon in boldface) and plasmid pTL7-1. The PCR reaction was performed using AccuPrime Taq DNA polymerase (1U) and accompanying buffer (Invitrogen). The amplification reaction was started with an initial denaturation of 2 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 58 °C and 1 min at 68 °C, with an additional cycle of 5 min at 68 °C. The amplification product was purified using the QIAquick PCR purification kit (Qiagen), after which this product was introduced into the pDONR201 vector via a Gateway BP recombination reaction in a vector:insert molar ratio of 1:2. After transformation of *E. coli* TOP10, recombinant cells were selected by plating on LB plates containing kanamycin (50 μg/mL), followed by overnight incubation at 28 °C. The PDF-encoding gene in pDONR201 was subsequently recombined to expression vector pBAD/*Myc*-His-DEST in a standard Gateway recombination reaction using LR clonase (Invitrogen) and a molar ratio of destination vector vs. entry vector of 1:2.4 (150:300 ng). After transformation of *E. coli* TOP10, recombinant cells were selected by plating on LB plates containing carbenicillin (100 μg/mL) followed by overnight incubation at 28 °C. Finally, a correct clone, as established by plasmid DNA isolation and restriction enzyme analysis was designated pBAD/Myc-His-DEST PDF_{wt}, or pBAD-PDF_{wt}.

Construction of EcPDF variant expression vector

The Kunkel method [14] that we used for site-directed mutagenesis of the *def* gene was performed according to the instruction manual of the Muta-Gene phagemid kit (Bio-Rad Laboratories GmbH, Munich, Germany). The *def* gene from *E. coli* K12 (EMBL accession number U00096, nucleotides 3,431,712–3,432,221) was cloned between the EcoRI and HindIII sites of phagemid pTZ18U [14], placing the gene under the control of the *lac* promoter [15]. The resulting phagemid was used for mutagenesis. A DNA segment encoding an octaglutamate tag between codons for Glu64 and Asn65 was obtained using the primer prPDF-Etag (5′-CGTCAC GGTCCTCTTCTTCTT CCTCCTCTTCCTCTTCCGAAACATC-3′, AvaII restriction site underlined). The oligoglutamate tag is encoded by a mixture of CCT and CTT codons (in bold) to prevent hairpin formation. For the construction of PDF_{short} the primer was prPDFstop (5′-GTTGTTTCA**CTTAAG** ACAGATAATCC-3′, AflII site underlined) which changes the CCG codon for Pro148 into a stop codon (TAA, in bold). After synthesis of dsDNA, this was used to transform *E. coli* JM109 (*ung*+) which contains uracil *N*-glycosylase for inactivating the uracil-containing template. The resulting PDF_{Etag}- and the PDF_{short}-encoding plasmids were respectively called pTL7-1 and pTL7-2. For expression, the mutated genes were recloned into the pBAD/*Myc*-His-DEST vector as described above, yielding pBAD-PDF_{Etag} and pBAD-PDF_{short}.

Cultivation and preparation of cell-free extract

$EcPDF_{wt}$, $EcPDF_{Etag}$ and $EcPDF_{short}$ were isolated from overproducing *E. coli* TOP10 cells transformed with the respective plasmids. Cells were grown at 28 °C for 14–16 h in 1.5 L LB medium containing 100 μg/mL carbenicillin. When the OD_{620} reached 0.6, PDF expression was induced by addition of 0.02% L-arabinose. Cells were harvested by centrifugation and about 12 g (wet weight) of cell paste was suspended in 40 mL buffer (20 mM Hepes/KOH, 100 mM KCl, pH 7.7, supplemented with 10 μg/mL catalase from bovine liver). After sonication at 0 °C, cell-free extract (CFE) containing 5–10 mg/mL of protein was obtained by centrifugation at 33,300*g* for 1 h at 4 °C.

Purification procedures for PDF

For purification by anion exchange chromatography, 1 mL of CFE was fractionated with a 1 mL Mono Q 5/50 GL column operated with HTK-buffer (20 mM Hepes/KOH, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 100 mM KCl, pH 7.7). PDF was eluted with 60 mL of a 0–1 M KCl gradient. Fractions with PDF activity were concentrated using Centriprep 30 kDa filters to 10 mg/mL of protein. Purified enzyme was stored at −20 °C.

This purification procedure was also scaled up. An amount of 44 mL of CFE (30 mg/mL total protein) was loaded on a HiLoad 26/10 Q Sepharose HP column equilibrated with HTK-buffer. Unbound proteins were washed off with 100 mL of the same buffer and PDF was eluted with 1 M KCl in HTK buffer, and concentrated by ultrafiltration to 10 mg/mL of protein.

For purification of by affinity column chromatography, CFE was prepared in HFC buffer (20 mM Hepes/KOH, pH 7.7, 100 mM KF, supplemented with 10 μg/mL catalase from bovine liver). An amount of 10 mL of CFE was fractionated using a 20 mL Met–Lys-Sepharose column that had been equilibrated with HFT-buffer (20 mM Hepes/KOH, pH 7.7, containing 100 mM KF and 0.2 mM TCEP) [11]. After washing, PDF was eluted with the same buffer containing containing 100 mM KCl and concentrated to 10 mg/mL.

Purification by ultrafiltration was tested with CFE from *E. coli* TOP10 cells expressing PDF_{wt}. using Centriprep or Centricon devices equipped with 50 or 100 kDa cutoff filters (YM-50 or YM-100, Millipore). Before loading, samples were centrifuged for 2 h at 16,100*g* and 4 °C, diluted 5-fold with buffer composed of 20 mM Hepes/KOH, pH 7.7, 100 mM KCl, and supplemented with 10 μg/mL catalase and 2 mM TCEP. Diluted material (3 mg/mL) was loaded and centrifuged for 2.5 h at 1000*g* and 4 °C.

For purification of PDF by gel filtration chromatography, cell-free extracts were diluted with standard dilution buffer (SDB), which was composed of 20 mM MOPS/NaOH, 100 mM NaCl, supplemented with 10 μg/mL catalase from bovine liver and 2 mM TCEP, pH 7.7, to a protein concentration of 5 mg/mL. Subsequently 100 μL samples of the extracts were chromatographed on a TSK-GEL $G2000SW_{XL}$ column (TOSOH Bioscience). The flow rate was 0.5 mL/min.

Enzyme and protein assays

Deformylase activity of PDF was routinely measured at 30 °C in a volume of 500 μL. Samples of 50 μL of PDF in SDB were mixed with 450 μL of substrate solution composed of 110 mM MOPS/NaOH buffer (pH 7.2), 300 mM NaCl, 0.1 mg/mL bovine liver catalase, and 5.5 mM of the substrate *N*-formyl-Met–Ala-OH. The dilutions of the enzyme were chosen such that approx. 10% of the substrate was converted in 15 min. After starting the reaction by addition of PDF, 100 μL aliquots were withdrawn from the reaction mixture every 5 min and added to 100 μL of 1 M phosphate buffer (H_3PO_4-NaOH, pH 2.66) to stop the reaction. Analysis of substrate and product was performed using HPLC.

Peptidase activity in samples was determined using a mixture of the following 8 dipeptides: H-Leu–Phe-OH, H-Phe–Leu-OH, H-Asp–Phe-OH, H-Phe–Asp-OH, H-Arg–Phe-OH, H-Phe–Arg-OH, H-Gly–Phe-OH, H-Phe–Gly-OH. An amount of 450 μL of a solution composed of 110 mM MOPS/NaOH buffer (pH 7.2), 300 mM NaCl, 0.1 mg/mL bovine liver catalase, and 0.625 mM of each of the 8 dipeptides, was mixed with 50 μL of different dilutions of PDF in SDB. After starting the reaction at 30 °C by the addition of PDF, 50 μL samples were withdrawn at various times and added to 150 μL methanol to stop the reaction. Analysis of substrates and product (L-phenylalanine) was performed using HPLC as described below.

Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA, Sigma) as standard protein. SDS–polyacrylamide gelelectrophoresis was performed according to standard protocols. Marker proteins are from the BenchMark Protein Ladder (Invitrogen), containing marker proteins of 220, 160, 120, 100, 90, 80, 70, 60, 50 (thick band), 40, 30, 25, 20 (thick band), 15 and 10 kDa).

PDF stability

The effect of several water miscible organic cosolvents [methanol, tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), *tert*-butanol, dimethyl sulfoxide (DMSO) and *N*-methyl-2-pyrrolidinone (NMP)] in a 10, 20 or 40% (v/v) concentration was measured by incubating diluted purified $EcPDF_{wt}$ and its variants at 30 °C in a solution containing 20 mM MOPS/NaOH, pH 7.7, 100 mM NaCl, 10 μg/mL bovine liver catalase, and 2 mM TCEP. To this mixture was added 450 μL of substrate solution composed of 110 mM MOPS/NaOH buffer, pH 7.2, 300 mM NaCl, 0.1 mg/mL bovine liver catalase and 5.5 mM of the substrate *N*-formyl-Met–Ala-OH. Samples were periodically withdrawn from the incubation mixture to measure the specific activity. The residual activity is reported as a percentage of the specific activity of the enzyme that was found when no cosolvent was added to the reaction mixture.

The temperature stability of the enzymes was analyzed by preincubating purified PDFs (30 μg/mL) for 60 min at temperatures between 4 and 60 °C in standard dilution buffer. After an incubation of 10 min on ice, 50 μL samples of the pretreated enzyme solutions were used to determine the remaining activity according to the standard assay at 30 °C.

HPLC analyses

HPLC was carried out using a stainless-steel analytical column (250 mm length, 4.6 mm ID) packed with Inertsil ODS-3 material, 5 μm particle size from Alltech Applied Science (Breda, The Netherlands). The flow rate was 1 mL/min. UV detection was performed at 40 °C at a wavelength of 210 nm. The injection volume was 5 μL.

N-formyl-For-Met–Ala-OH and H-Met–Ala-OH were analyzed using the following gradient of acetonitrile in 10 mM H_3PO_4: t = 0–4 min, 0.1% (v/v) acetonitrile isocratic; t = 4–15 min, 0.1–50% acetonitrile linear increase; t = 15–15.1 min, 50–0.1% linear decrease; t = 15.1–20 min, 0.1% acetonitrile isocratic (retention times: *N*-formyl-Met–Ala-OH = 13.54 min, Met–Ala-OH = 5.39 min).

The peptides H-Leu–Phe-NH_2, *N*-formyl-Leu–Phe-NH_2, and *N*-formyl-Tyr–Leu–Phe-NH_2 were analyzed using the following gradient of acetonitrile in 10 mM H_3PO_4: t = 0–5 min, 2.5% (v/v) acetonitrile isocratic; t = 5–20 min, 2.5–75% acetonitrile linear increase; t = 20–20.1 min, 75–2.5% linear decrease; t = 20.1–27 min, 2.5% acetonitrile isocratic (retention times: H-Leu–Phe-NH_2 = 12 min, N-formyl-Leu–Phe-NH_2 = 16.81 min, N-formyl-Tyr–Leu–Phe-NH_2 = 19.46 min.

For peptidase activity measurements, the production of L-phenylalanine was analyzed using the following gradient of acetonitrile in 10 mM H_3PO_4: t = 0–15 min, 0–30% acetonitrile linear increase; t = 15–15.1 min, 30–0% acetonitrile decrease; t = 15.1–20 min, 0% acetonitrile isocratic.

Chemo-enzymatic synthesis of N-formyl-Tyr–Leu–Phe-NH_2 using $EcPDF_{Etag}$

N-formyl-Leu–Phe-NH_2 was synthesized as follows: 7.9 g (49.7 mmol) *N*-formyl-Leu-OH and 10 g (61.0 mmol, 1.23 equiv) H-Phe-NH_2 were dissolved in 200 mL of water containing 0.4 mM NaCl and 15 mM $CaCl_2$ keeping the pH at 6 by addition of NaOH (20% w/v). Then, 4 g of thermolysin (Sigma) was added and the mixture was stirred for 30 h at ambient temperature. The precipitate was isolated by filtration and washed with 50 mL of water. The identity of *N*-formyl-Leu–Phe-NH_2 was confirmed by NMR analysis and its purity (>96%) was determined by HPLC analysis.

PDF-catalyzed deprotection of *N*-formyl-Leu–Phe-NH2 was performed as follows. To a solution of 55 mM *N*-formyl-Leu–Phe-NH_2 in 260 mM aqueous MOPS-NaOH buffer containing 675 mM NaCl and 0.1 g/L of catalase (pH 7.2) was added purified PDFEtag (obtained using ion-exchange chromatography) to a final concentration of 5 μM. The reaction mixture was stirred at 28 °C for 46 h. The product was isolated in 80% yield by extraction with ethyl acetate at pH 9.5 and subsequent evaporation of the ethyl acetate phase in vacuo. The identity of Leu–Phe-NH_2 was confirmed by NMR spectroscopy and its purity was checked by HPLC (>98%).

For the synthesis of N-formyl-Tyr–Leu–Phe-NH_2, a 4 mL aqueous solution of 0.334 g N-formyl-Tyr-OH and 0.390 g H-Leu-Phe-NH_2 in H_2O containing 0.4 mM NaCl and 15 mM CaCl2 was prepared and 0.1 g thermolysin (Sigma) was added. The mixture was incubated at ambient temperature for 3 days. The product was isolated by filtration and washed with 10 mL water and 5 mL diethyl ether. The identity of *N*-formyl-Tyr–Leu–Phe-NH_2 was confirmed by NMR and its purity (>65%) was checked by HPLC.

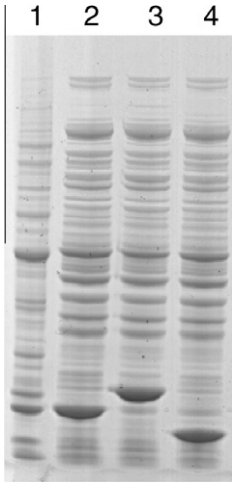

Fig. 1. SDS–PAGE analysis of induced *E. coli* cells overexpressing EcPDF variants. Lanes: 1, reference protein ladder; 2, CFE of *E. coli* cells overexpressing $EcPDF_{wt}$; 3, idem $EcPDF_{Etag}$; 4, idem $EcPDF_{short}$.

Results and discussion

Production of PDF

To obtain an expression construct for $EcPDF_{wt}$ and its variants, the *E. coli def* gene, coding for $EcPDF_{wt}$, or variants thereof were cloned into pBAD/*Myc*-His-DEST via standard molecular biology procedures. This resulted in a high-level overexpression in *E. coli*. of $EcPDF_{wt}$. A construct in which codons for eight glutamates were introduced between codons for Glu64 and Asn65 was constructed and expressed with the same vector. A shorter variant of *E. coli* PDF (see below) was obtained by changing the codon for Pro148 by a stop codon (TAA, in bold). All three PDFs were well overexpressed in soluble form using the same vector (Fig. 1). The specific activity of PDFwt in CFE was 229 U/mg as measured with the standard assay, compared to 16 U/mg in a non-overexpressed CFE. Levels in CFE for $EcPDF_{Etag}$ and $EcPDF_{short}$ were 180 U/mg and 215 U/mg, respectively. The PDF proteins were produced in *E. coli* at levels of 10–20% of the total soluble protein recovered in CFEs.

Initially, membrane filters with different pore sizes and filter setups were tested for separating PDF from other proteins. However, even when CFEs were loaded on a 100 kDa Centriprep filter, only about 10% of the deformylase activity was found in the filtrate, both with $EcPDF_{wt}$ and $EcPDF_{short}$. Even pure protein obtained by affinity chromatography did not easily pass the 30 or 50 kDa cutoff filters, suggesting protein aggregation or interaction with the filter material. The use of cross-flow filtration or additives such as salt (0.1 or 1 M NaCl) or betaine [16,17] did not improve the results. Thus, even though PDF is a small protein, ultrafiltration appeared ineffective, possibly due to fouling of membrane material.

Purification of E. coli PDF via affinity chromatography

For purification of $EcPDF_{wt}$, we first tested the single-step procedure using a Met–Lys-Sepharose affinity column as described in Materials and Methods. In the Met–Lys-Sepharose matrix, the ligand is bound via the ε-NH_2 group of the Lys side-chain to the *N*-hydroxysuccinimide-activated Sepharose. The method is based on the binding of active PDF to the Met–Lys-Sepharose affinity matrix in the presence of fluoride ions, which significantly increase the affinity of $EcPDF_{wt}$ for small peptides, most likely because fluoride mimics formate.

The result of a typical purification experiment starting from ca. 15 g (wet weight) *E. coli* TOP10 cells containing pBAD-PDF_{wt} is given in Table 1. The specific activity of the purified PDF towards the substrate *N*-formyl-Met–Ala-OH was 1880 U/mg protein at pH 7.2 and 30 °C. This is somewhat higher than the 1170 U/mg reported for a homogeneous preparation of PDF(Fe) [18]. Purified PDF obtained this way served as a reference when measuring the amidase and peptidase content of various enzyme samples.

Table 1
Purification of $EcPDF_{wt}$ from *E. coli* TOP10 using Met–Lys-Sepharose affinity chromatography.

Purification step	Activity ($\times 10^3$ U)[a]	Protein (mg)	Deformylase (U/mg)[a]	Recovery (%)	Purification (fold)	Peptidase (U/mg)[b]	Deformylase peptidase. ratio
Affinity chromatography							
CFE	14.7	64.5	229	100	1	0.23	995
Met–Lys-Sepharose	8.4	4.5	1,880	57	8.2	0.002	$1 \cdot 10^6$

[a] Deformylase activities measured with *N*-formyl-Met–Ala-OH as the substrate.
[b] Peptidase activity; was determined by peptide bond hydrolysis rates of a mixture of eight phenylalanine-containing dipeptides was used (see Materials and methods).

To enable rapid determination of amidase and peptidase activity, enzyme samples were incubated with a mixture of 8 different dipeptides, each containing a Phe at the N- or at the C-terminal position. The increase of purity of PDF containing samples was determined by calculating the ratio of the deformylase activity and peptidase activity, and comparing this to the same ratio found for pure PDF. This calculation showed that in PDF samples purified by affinity chromatography the level of contamination by peptidases was reduced over thousand fold as compared to CFE. However, in view of the costs of the affinity matrix and the use of fluoride, this purification method is not attractive for use on industrial scale and consequently we explored alternative and more cost-efficient purification procedures for PDF.

Purification of PDF via ultrafiltration

Data on the use of ultrafiltration (UF) for protein purification purposes are limited [19,20]. Since EcPDF is a monomeric globular protein of about 19 kDa [21] whereas most proteases are multimers of monomers with a size of about 60 kDa [22], ultrafiltration could offer a simple and cost effective way of separating PDF from amidases and peptidases. For testing ultrafiltration, both wt *E. coli* PDF was used as well as a smaller variant ($EcPDF_{short}$) that lacks residues 148–169 from the C-terminal α-helix of the EcPDF. It is known that the last 18 residues of EcPDF are disordered in the X-ray structure and dispensable for activity [23]. $EcPDF_{short}$ is a protein of 16.7 kDa.

Purification of PDF via ion-exchange chromatography

The use of ion-exchange chromatography (IEC) as a cheap and easily scalable protein purification method for separating PDF in a single chromatographic step from amidases and peptidases was attempted with a Mono Q 5/50 GL column (Table 2). The specific activity of the purified $EcPDF_{wt}$ and the recovery were unsatisfactory with a yield of only 19%. Moreover, the level of contamination due to the presence of amidases and peptidases in the sample was only reduced by a factor of 2, when the fractions containing the highest levels of PDF activity were pooled and tested.

To obtain better separation, the use of a purification tag was considered. Most commercial tags used in research, such as a hexahistidine tag, are too expensive for large-scale preparation of proteins because they require expensive resins. Protein-based tags, such as maltose-binding protein, are large as compared to the size of PDF and require expensive processing enzymes for removal. The fusion of a small tag that introduces multiple charged amino acids could help separation by classical ion exchange chromatography. It was observed that a tag consisting of six arginines fused to the C-terminal end of human urogastrone could act as an ion-exchange tag [24,13]. Other positively and negatively charged tags composed of multiple arginine, glutamate or aspartate residues have been constructed [25–30]. More general purification tags providing different interaction possibilities have also been proposed [31].

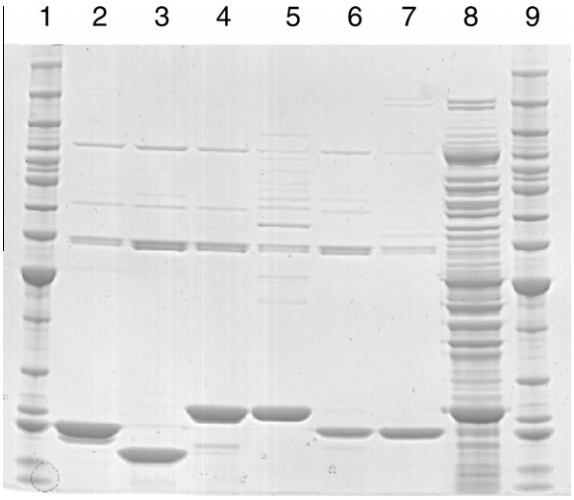

Fig. 2. SDS–PAGE analysis of purified EcPDF variants using IEC and affinity chromatography. Lanes: (1) reference proteins; (2) $EcPDF_{wt}$ purified via affinity chromatography; (3) $EcPDF_{short}$ purified via affinity chromatography; (4) $EcPDF_{Etag}$ purified via affinity chromatography; (5) $EcPDF_{Etag}$ purified via IEC; (6) $EcPDF_{short,Etag}$ purified by affinity chromatorgaphy; (7) idem, $EcPDF_{short,Etag}$; (8) CFE of $EcPDF_{Etag}$; (9) reference proteins.

In view of these results, we inserted an octaglutamate tag (Etag) in the flexible loop that is present between β-strands C and D (residues 61–66) of PDF (PDB code 1BS6). It has been reported that this CD loop has to be flexible but has no role in activity and/or substrate binding. Moreover, it is not conserved and its composition and length varies in different PDF types [32,33]. The point of insertion was chosen after codon 192 (TCG) which encodes Glu64 in the wild-type sequence. The construct was verified by sequencing and expressed in *E. coli* TOP10 yielding the desired $EcPDF_{Etag}$. The overexpression of this new PDF variant was similar to what was achieved with $EcPDF_{wt}$ (20% of the total protein) and the deformylase activity of the $EcPDF_{Etag}$ was only slightly affected by the presence of the charged moiety. After purification using Met–Lys-Sepharose affinity chromatography (Fig. 2), the specific activity of $EcPDF_{wt}$ towards the substrate *N*-formyl-Met–Ala-OH was 1800 U/mg while with $EcPDF_{Etag}$ an activity of 1300 U/mg was found.

Unlike the wild-type enzyme, the E-tag containing protein could easily be separated from amidases and peptidases by ion-exchange chromatography (Table 2, Fig. 2). The short PDF variant equipped with an E-tag ($EcPDF_{short,Etag}$) could also be purified this way (Fig. 2). When using a Mono Q 5/50 GL column, more than 70% of the PDF activity was found back after IEC and the amount of amidases and peptidases in the purified sample was reduced by a factor of 1500. A similar purity was found when $EcPDF_{Etag}$

Table 2
Purification of $EcPDF_{Etag}$ from CFE of *E. coli* TOP10 containing pBAD-PDF_{Etag} using ion-exchange columns.

Purification step	Activity (×103 U)[a]	Protein (mg)	Deformylase act. (U/mg)[a]	Recovery (%)	Purification (fold)	Peptidase act. (U/mg)[b]	Deformylase/ peptidase. ratio
Mono Q 5/50 GL column							
CFE	0.8	4.5	180	100	1	0.16	1120
IEC column	0.6	0.6	970	73	5	$6{\cdot}10^{-4}$	$1.6{\cdot}10^{6}$
HiLoad 26/10 ion-exchange column							
CFE	21	1400	156	100	1	0.19	821
HiLoad 26/10	14	150	884	64	5.5	$8{\cdot}10^{-4}$	$1.1{\cdot}10^{6}$

[a] Deformylase activities measured with *N*-formyl-Met–Ala-OH as the substrate.
[b] Peptidase activity was determined by measuring the release of L-Phe from a mixture of eight phenylalanine-containing dipeptides (see Materials and methods).

was purified on larger scale from CFE using a HiLoad 26/10 column. This procedure allowed the isolation, in a single step, of 150 mg of pure $EcPDF_{Etag}$ from 44 mL of CFE obtained from a 1 L culture.

The results show that IEC in combination with the use of an engineered PDF carrying a charged octaglutamate tag is a suitable method for the removal of amidases and peptidases from EcPDF to be used in peptide deformylation.

Suitability of $EcPDF_{Etag}$ for deprotection of enzymatically synthesized N-formyl-dipeptides

The purified $EcPDF_{Etag}$ was subsequently used in a reaction containing 1.2 mM *N*-formyl-Leu–Phe-NH_2. The mixture was incubated under standard reaction conditions (pH 7.2, 30 °C, 8 μg/mL $EcPDF_{Etag}$). After 4 h, full deformylation of the substrate was obtained without any detectable peptide bond hydrolysis. When the deformylation reaction was carried out with CFE, extensive peptide bond hydrolysis occurred, most likely due to leucine aminopeptidase activity produced by the *E. coli* host.

Polar organic solvents, such as dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), methanol (MeOH), *N*-methyl-2-pyrrolidinone (NMP), tetrahydrofuran (THF) and *tert*-butanol are often utilized as cosolvents to increase peptide solubility [34]. Therefore, the stability of $EcPDF_{wt}$, $EcPDF_{short}$ and $EcPDF_{Etag}$ was tested by determining activities in the presence of different concentrations of cosolvents. Complete inactivation of these PDFs was observed when the enzymes were incubated with 10% (v/v) NMP and THF. The effect of DMF and DMSO was not as detrimental, but remaining activities were very low for all three enzymes, even when only 10% cosolvent was used (Fig. 3A and B). With methanol, the loss of activity was somewhat less, but the residual activity was still low since only 20% of the initial activity was left in the presence of 20% (v/v) methanol (Fig. 3). Nevertheless, the data show that there is little difference in enzyme stability between the $EcPDF_{wt}$ and the two engineered variants.

In order to demonstrate the applicability of the purified $EcPDF_{Etag}$ in chemo-enzymatic peptide synthesis, we synthesized the tripeptide *N*-formyl-Tyr–Leu–Phe-NH_2 using two thermolysin-catalyzed coupling steps and one PDF-catalyzed deformylation step according to the scheme in Fig. 4. The formyl group can be conveniently introduced on single amino acids using formic acid and acetic acid anhydride. First, a dipeptide was prepared by coupling the formyl-protected amino acid *N*-formyl-Leu with H-Phe-NH_2 using thermolysin (Fig. 4, step a). The formylated dipeptide precipitated from the reaction mixture and could be isolated by filtration. Subsequently, it was deprotected with $EcPDF_{Etag}$ (Fig. 4, step b), which was successfully completed even though the formyl group needed to be removed from a Leu residue instead of the Met residue that is present on the *N*-terminus of PDF's natural substrates. Deprotection by PDF resulted in complete removal of the formyl

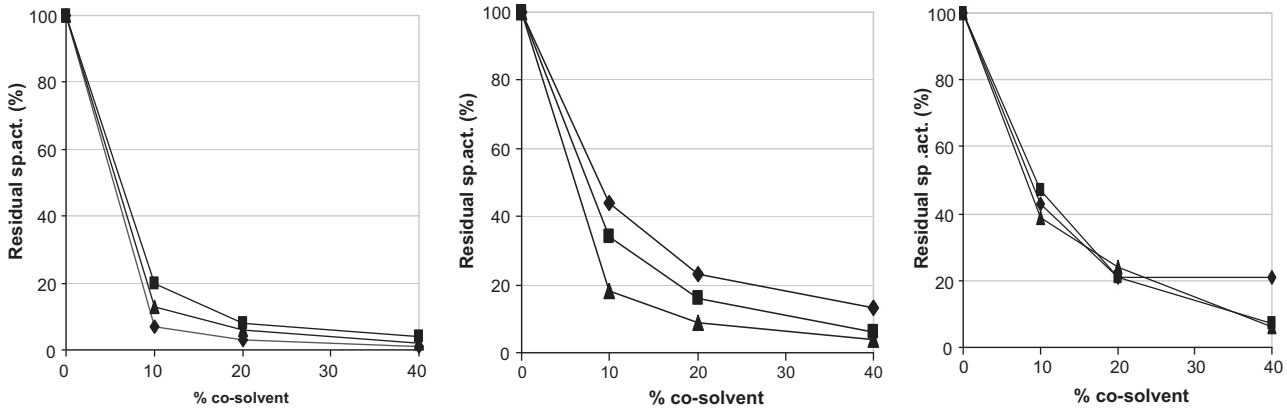

Fig. 3. Effect of organic solvents on PDF activity. Panels: A, *N,N*-dimethylformamide; B, DMSO; C, methanol. Symbols: $EcPDF_{wt}$; $EcPDF_{Etag}$; $EcPDF_{short}$.

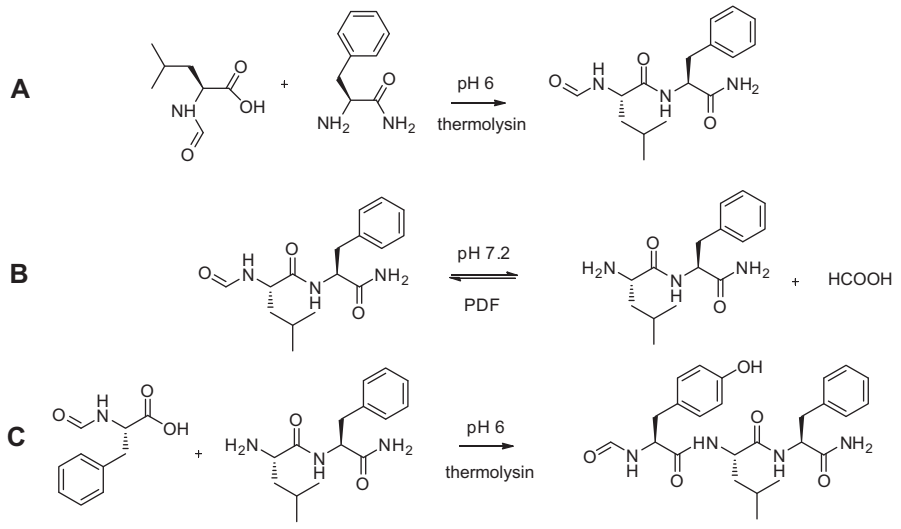

Fig. 4. Reaction scheme for the synthesis of *N*-formyl-Tyr–Leu–Phe-NH_2 starting from amino acid building blocks.

group, without any detectable peptide bond hydrolysis, indicating that amidases and peptidases were absent.

After isolation of H-Leu–Phe-NH_2 using extraction with ethyl acetate at pH 9.5, the purity of the product was >98% with a final yield of 80%. The deprotected Leu–Phe-NH_2 dipeptide was subsequently coupled to *N*-formylated tyrosine applying thermolysin as catalyst (Fig. 4, step c). This yielded the desired tripeptide *N*-formyl-Tyr–Leu–Phe-NH_2.

In conclusion, we have engineered and overexpressed in *E. coli* TOP10 a variant of *E. coli* PDF with a octaglutamate tag inserted into a flexible surface loop. This *E. coli* PDF_{Etag} was subsequently purified from the overproducing strain by a single step of anion exchange column chromatography. This purification method is easily scalable and requires only cheap materials, making it industrially attractive. Although not completely pure, the enzyme lacked most of the amidase and peptidase that is present in CFE and is detrimental for peptide synthesis applications. The enzyme appeared to be suitable for the application in peptide synthesis since it allows a mild and selective deprotection of *N*-formyl peptides without detectable peptide bond hydrolysis.

Database links

Uniprot: http://www.uniprot.org/uniprot/P0A6K3.

RSCB: http://www.rcsb.org/pdb/explore/explore.do?structureId=1DFF.

RSCB: http://www.rcsb.org/pdb/explore/explore.do?structureId=1BS5.

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